1952, v83, 487.

14. Chatterjee, S. N., Sarkar, J. K., Proc. 5th Int'l Congress of Electron Microscopy, S. Breese, ed., Academic Press, N. Y., 1962, v2.

15. Britten, R. J., Roberts, R. B., Science, 1960, v131, 32.

16. Sever, J. L., J. Immunol., 1962, v88, 320.

17. Kempe, C. H., Diagnostic Procedures for Virus and Rickettsial Diseases, T. Francis, Jr., ed., APHA, New York, N. Y., 2nd ed., 1956.

18. McCrea, J. F., Ann. N. Y. Acad. Sci., 1960, v83, 692.

19. Knight, C. A., J. Exp. Med., 1946, v83, 281.

20. Frommhagen, L., Knight, C. A., Freeman, N. K., Virology, 1959, v8, 176.

21. Kates, M., Allison, A. C., Tyrell, D. A. J., James, A. T., Cold Spring Harbor Symposium, 1962, v21, 292.

22. Stenback, W. A., Durand, D. P., Virology, 1963, v20, 545.

23. Gaush, C. R., Youngner, J. S., ibid., 1963, v19, 573.

24. Ackermann, W. W., Loh, P. C., Ann. N. Y. Acad. Sci., 1960, v88, 1298.

25. Gaush, C. R., 1963 Ph. D. Thesis, U. Pittsburgh (L.C. Mic. No. 63-2425). University Microfilms, Ann Arbor, Mich.

26. Loh, P. C., Riggs, J. L., J. Exp. Med., 1961, v114, 149.

27. Oda, M., Virology, 1963, v20, 552.

Received October 9, 1964. P.S.E.B.M., 1965, v118.

Further Evidence of a High Degree of Genetic Homology Between *H. influenzae* and *H. aegyptius.** (29935)

GRACE LEIDY, IRIS JAFFEE AND HATTIE E. ALEXANDER

Babies Hospital and Department of Pediatrics, Columbia University College of Physicians and Surgeons, New York

DNA-mediated interspecific transformations have been reported for a number of bacterial "species" or groups(1) and are interpreted as evidence of some degree of genetic homology among the groups which exchange genetic material (DNA). Among "species" of Hemophilus, the heterospecific to homospecific transformation ratio is low for *H. influenzae* populations exposed to *H. parainfluenzae* or *H. suis* DNA and for *H. parainfluenzae* cells exposed to *H. influenzae* DNA (2). On the other hand, this ratio may approach unity at times when *H. influenzae* and *H. aegyptius* populations are considered(3); a high degree of genetic homology for these 2 "species" is suggested. This conclusion was based on data from experiments in which streptomycin (S) resistance was used as a genetic marker. The present study extends this analysis of interspecific transformation in *H. influenzae* and *H. aegyptius* to include resistance to novobiocin (NV) as a genetic marker. NV resistance is linked to S resistance in *H. influenzae*(4). The data presented show a high heterospecific to homospecific transformation ratio for these markers in members of the 2 "species" and indicate that NV resistance is linked to S resistance in *H. aegyptius* also. The data also show a "species" difference in sensitivity to NV which is reflected in the level of NV resistance conferred by the heterologous species NV resistance transforming factor.

Materials and methods. Recipient populations. Strains R*b* and R*d* *H. influenzae* and 15 and 181*a* *H. aegyptius*(3). We are indebted to Dr. Margaret Pittman for *H. aegyptius* strains.

DNA donor populations. 1) *H. influenzae* R*d* S 1000 NV 5; nonencapsulated variant derived from type *d*; resistant to 1000 μg S per ml and 5 μg NV† per ml. A NV resistant population was selected first by seeding 5×10^7 sensitive cells in pour plate preparations of Levinthal agar containing 2 μg NV per ml. A single colony which formed in this environment within 48 hours incubation was

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selected and a subclone isolated after 2 subcultures in the absence of NV. This population, by the streak plate method, grew well in the presence of 5 μg NV per ml, and showed a 90% or greater reduction in number of colonies in the presence of 10 μg NV per ml. Subsequently, from the above population resistant to NV, a spontaneously occurring mutant population resistant to at least 1000 μg S per ml was selected.

2) *H. aegyptius* 15 S 1000 NV 0.4, resistant to 1000 μg S per ml and 0.4 μg NV per ml. A NV resistant mutant was first selected by spreading approximately 10^8 cells on the surface of Levinthal agar containing NV in concentrations in the range of 0.1 to 1.0 μg per ml. Only 1 colony emerged (0.4 μg per ml) within a 48- to 96-hour incubation period. On subculture, growth in the presence of 0.4 μg was slower in relation to the control lacking NV; in the presence of 1 μg NV per ml there were fine colonies, less than 50% of the control, after 48 to 96 hours incubation. No growth occurred in the presence of 2 μg NV per ml. A spontaneously occurring S resistant mutant was isolated from this population resistant to 0.4 μg NV per ml by seeding approximately 5×10^9 cells in each of 6 pour plate preparations containing 1000 μg S per ml and selecting one of 8 colonies which emerged with incubation. When the S resistant culture was examined for its sensitivity to NV by the streak plate method, growth comparable to that of the control was manifest within 24 hours at a concentration of 0.2 μg NV per ml and only a slightly reduced number of smaller colonies in the presence of 0.4 μg NV per ml. There was no growth at a concentration of 2 μg NV per ml.

Medium used for growth. "Elev." broth (5) was used for the growth of competent populations. Levinthal agar and broth were otherwise used(6).

Preparation of competent populations. Recipient cells were propagated by the method of Goodgal and Herriott(5); details of the method as used by us have been reported(7; "Saline control").

Methods used to measure transformants. Competent populations after a 10- or 15-minute exposure to the transforming agents (DNA) were seeded either in Levinthal broth or agar and incubated at 37°C for 2 hours (strains R*d* and R*b* *H. influenzae*) or 2½ to 3 hours (strains 15 and 181*a* *H. aegyptius*) to permit expression of the new trait. Populations seeded in agar were then layered with an approximately equal volume of agar in which the antibiotics were incorporated in twice the concentration indicated (agar overlay method). Aliquots of the treated populations with continued incubation in broth for 2 to 3 hours after DNA treatment were seeded in pour plate preparations of antibiotic containing agar (pour plate method). The latter method was used to insure simultaneous and uniform exposure of transformants to particular concentrations of NV. The former method (agar overlay) permitted a more accurate measurement of the number of transformants since progeny of transformants were excluded from measurement.

Exposure to DNA was terminated by deoxyribonuclease (DNAase, 1 μg per ml). Competent populations not exposed to the DNA or exposed to DNAase inactivated DNA served as controls in all experiments. Cell saturating concentrations of DNA (approximately 0.5 or 1 μg per ml) were used except for linkage relationship studies.

In vitro *sensitivity of natural strains of* H. aegyptius *and* H. influenzae *to NV.* Six- to seven-hour subcultures in the late logarithmic (or early stationary) phases of growth were used. Population density varied from 9.0×10^8 to 1.8×10^9 colony forming units per ml. Two different procedures were used to measure the concentration which prevents growth of more than 99% of the population: 1) The antibiotic in varying concentrations was incorporated in agar for plate preparations and approximately 10^6 cells were streaked on the surface of the agar, and 2) Liquid agar preparations containing the same varying concentrations of NV were seeded in duplicate with 0.5 ml of a 10^{-7} dilution of the population for pour plate preparations. Approximately 80 to 175 colony forming units were seeded for each culture.

Experimental results. To obtain additional information on the degree of genetic homology between *H. influenzae* and *H. aegyptius,* experiments were designed to answer the following questions: 1) Is there a natural "spe-

TABLE I. Comparison of Sensitivity to Novobiocin (NV) of Natural Strains of *H. influenzae* and *H. aegyptius*.

Strain examined	Agar plate method used	μg NV per ml							
		0	.01	.025	.05	.1	.2	.3	.5
H. influenzae									
R*d*	Streak*	+	+	+	+	+	0	—	0
	Pour†	157	—	—	171	172	41	0	—
R*b*	Streak	+	+	+	14	1	0	—	0
	Pour	105	—	118	58	0	—	—	—
H. aegyptius									
15	Streak	+	11	0	0	0	—	—	—
	Pour	81	83	0	—	—	—	—	—
181*a*	Streak	+	28	0	0	0	—	—	—
	Pour	171	158	0	—	—	—	—	—

* Streak plate: Approximately 10^6 cells seeded. + = growth comparable to control; 0 = no growth. Numbers = single colonies.

† Pour plate: No. of colony forming units per ml dilution used.

cies" difference in sensitivity to novobiocin (NV)? 2) Is the heterospecific : homospecific transformation ratio for NV resistance of the same order of magnitude as that found for streptomycin (S) resistance in *H. influenzae* and *H. aegyptius*? 3) Are the NV and S resistance transforming factors linked in *H. aegyptius*, as demonstrated in *H. influenzae*? 4) If linked, is the degree of linkage comparable in homospecific and heterospecific transformation?

In vitro *sensitivity of natural strains of* H. influenzae *and* H. aegyptius *to novobiocin.* Demonstration in preliminary experiments that the transforming agent derived from strain R*d* *H. influenzae* resistant to 5 μg NV per ml conferred a much lower level of resistance in *H. aegyptius* populations than in *H. influenzae* populations, led us to compare the sensitivity to NV or natural strains of these two "species." The methods used are described under "Materials and methods." The data in Table I show that strain R*b* *H. influenzae* is approximately 3- to 4-fold more sensitive to NV than strain R*d*, but that the *H. influenzae* strains are approximately 4- to 10-fold more resistant to NV than the *H. aegyptius* strains. Although the data suggest that cells tested for their NV sensitivity on the surface of agar are slightly more sensitive than those tested in deep agar, subsequent periodic examination by the two methods of populations transformed to NV resistant, has revealed a variability which precludes such a generalization. It is of interest that no "species" difference in sensitivity to *streptomycin* was demonstrated by the streak plate method for the natural strains listed in Table I.

Reflection by heterologous DNA transformation of natural differences in sensitivity to NV of H. influenzae *and* H. aegyptius. The natural "species" differences in sensitivity to NV influence the level of NV resistance conferred in interspecific transformation as shown in Table II. The transforming agents, designated TA R*d* S NV 5 and TA 15 S NV 0.4, were derived from populations resistant both to S (1000 μg per ml) and to NV (in concentrations indicated) and were presumably of single step mutational origin.

Natural populations of R*d* *H. influenzae* and strain 15 *H. aegyptius* sensitive to S and NV were treated separately with the DNA from the homologous and heterologous resistant "species" (TA R*d* S NV 5 and TA 15 S NV 0.4), incubated to allow expression of the new phenotypes (Materials and methods), and then examined for evidence of transformation to S and NV resistance by seeding in media containing NV alone and S alone (pour plate method).

The data (Table II) show that the DNA from the *H. aegyptius* population (TA 15 S NV 0.4) confers resistance to 0.2 μg to 0.4 μg NV per ml in members of the homologous "species," but a much higher level (approximately 3 μg to 5 μg per ml) in the *H. influenzae* populations. Transformants of R*d* *H. influenzae* which formed colonies in 0.5 μg NV per ml were resistant to at least 3 μg per ml. The conference of the higher level

TABLE II. Influence of Natural Differences in Sensitivity to NV on Degree of Resistance Conferred by Heterospecies DNA.

Recipient population	Transforming agent (DNA)	Transformants per ml† (μg NV or S per ml)											
		NV											S
		.1	.2	.4	.5	1	2	3	5	10	15	20	10‡ or 100
H. influenzae													
Rd	15 S* NV .4				600	700		610	540	270	0		550
	Rd S NV 5				2600	2500		2400	2500	2200	310	0	1700
Rb	15 S NV .4				150	150		120	1	0	0		80
	Rd S NV 5				210	210		140	70	2	0		91
H. aegyptius													
15	15 S NV .4	530	550	350	0	0							470
	Rd S NV 5	260	290	220	35	5	0						130
181*a*	15 S NV .4	480	420	370	0	0							310
	Rd S NV 5	160	160	180	30	8	0						90

* S = streptomycin resistance to at least 1000 μg per ml for all strains used as sources of DNA.
† Initial population sizes = Rd = 2.5×10^5; 15 = 4.0×10^5; Rb = 2.2×10^5; 181*a* = 5.7×10^5.
‡ 10 μg per ml for *H. aegyptius* strains; 100 μg per ml for *H. influenzae* strains.

of resistance in the *H. influenzae* populations by TA 15 S NV 0.4 is not due to selection of a proportion of DNA molecules (or parts) with the potential of conferring the higher levels, since the population used as the DNA source was virtually 100% sensitive to 1 or 2 μg NV per ml. For example, 0.1% of the population formed colonies in 1 μg NV per ml after 72 hours incubation; no colonies emerged in the presence of 2 μg NV per ml.

The action of the transforming agent from the *H. influenzae* strain (TA Rd S NV 5) in general parallels that from the *H. aegyptius* population (TA 15 S NV 0.4); a low level of resistance (0.2 μg to 0.4 μg NV per ml) is conferred in the *H. aegyptius* populations, but a higher level (5-fold or greater) in the *H. influenzae* populations. A difference in the action of the two transforming agents is suggested by the higher proportion of transformants which formed colonies in the highest concentrations of NV when the DNA from the *H. influenzae* strain (Rd S NV 5) was the transforming agent. Colonies formed from transformed cells in all the treated recipient populations inoculated into the higher NV concentration ranges required a longer period of incubation before gross appearance than those of less critical concentrations of NV; moreover, these colonies generally showed a greater degree of resistance when tested at the higher concentrations than those which formed in the presence of the lower concentrations.

Resistance to at least 1000 μg S per ml was conferred in all the receptor populations by the heterologous as well as homologous DNA. Ten μg per ml (*H. aegyptius* strains) and 100 μg per ml (*H. influenzae* strains) were used to measure the S transformants in these experiments. Sampling of colonies which formed in the concentration of S used to detect transformants (10 μg per ml for *H. aegyptius* and 100 μg ml for *H. influenzae*) were shown on reexamination to be resistant to 1000 μg per ml.

The activity of transforming agents derived from 2 other independent mutant populations of Rd *H. influenzae* resistant to 5 μg NV per ml, one other strain of *H. aegyptius* 15 resistant to 0.4 μg NV per ml, and one of strain 181*a* (*H. aegyptius*) resistant to 0.4 μg NV per ml, confirmed the "species" dependent pattern of interspecific transformation to NV resistance shown in Table II. Also, the DNA derived from *H. aegyptius* 15 cells, in which resistance to 0.2 to 0.4 μg NV per ml was conferred by the *H. influenzae* DNA, conferred a much higher level of NV resistance (5 to 10 μg NV per ml) in *H. influenzae* than in *H. aegyptius* populations (0.2 to 0.4 μg per ml).

The data presented in Table II suggest an influence of the natural sensitivity to NV which distinguishes the members of the 2 "species" examined, since degree of resistance conferred by the heterologous "species" DNA is correlated with degree of natural

sensitivity of the receptor population and not degree of resistance of the DNA donor. This correlation between the natural sensitivity of the receptor population and the level of NV resistance conferred by DNA is shown for members within a "species" as well. For example, studies showed that strain R*b* *H. influenzae* is more sensitive to NV than strain R*d* (Table I); this difference is reflected in the level of NV resistance conferred in homospecific as well as heterospecific transformation (Table II). It is of interest that the DNA from a strain of R*d* resistant to 0.5 μg NV per ml, but not 2 μg per ml conferred a comparable level (0.5 μg per ml) of NV resistance in R*d* (*H. influenzae*) sensitive populations but a 10-fold lower level (0.05 μg per ml) in *H. aegyptius* (strain 15) populations.

Experiments designed to confer the factor(s) responsible for the greater natural resistance of *H. influenzae* populations in *H. aegyptius* populations have failed. The transforming agent derived from a strain of R*d* *H. influenzae* resistant to streptomycin but sensitive to NV failed to confer resistance to concentrations of NV as low as 0.05 μg per ml in sensitive *H. aegyptius* (strain 15) populations.

In an earlier study(3) using the S resistance (1000 μg per ml) transforming factor, a high (0.2 to 1) heterospecific to homospecific transformation ratio was found for members of *H. influenzae* and *H. aegyptius*. The data of Table II show a similar high ratio (0.2 to 0.7) when the "species" are transformed to NV resistance. Data derived from experiments which used the agar overlay method provided a comparable ratio. The results of these experiments support our earlier proposal that *H. influenzae* and *H. aegyptius* are closely related. The ratio of the number of cells transformed to NV resistant by heterologous DNA to the number transformed by homologous DNA, again suggests a high degree of genetic homology.

Linkage of genetic determinants of S and NV resistance in H. aegyptius. Goodgal has reported linkage of NV and S resistance determinants in *H. influenza*(4). It was, therefore, of interest to extend our examination of the genetic homology of these 2 organisms by determining whether the genetic control of resistance to S and NV are linked in *H. aegyptius* and to compare the degree of linkage in the 2 "species" by heterospecific and homospecific transformation.

To explore linkage of S and NV resistance transforming factors, the natural *H. aegyptius* strain 15 was examined for the frequency of transformants which formed colonies in the presence of both antibiotics after exposure to a mixture of singly marked DNAs (TA 15 S 1000 and TA 15 NV 0.4) and to the DNA from cells which carried both resistance factors within the same cell (TA 15 S NV 0.4). The former transforming agents were each derived from a different population of *H. aegyptius* 15 cells: one was resistant to 0.4 μg NV per ml and sensitive to S, and the other, resistant to 1000 μg S per ml and sensitive to NV.

Ten μg S and 0.1 μg NV per ml were used to select for linked transformants in the *H. aegyptius* population. These concentrations were adopted to reduce the possibility of a synergistic type of action of the 2 antibiotics which was encountered in early studies of the interspecific linkage relationship in *H. aegyptius*, when 0.4 μg NV and 1000 μg S per ml were used together to measure the linkage relationship. Although transformants which formed colonies in the presence of 0.4 μg NV per ml alone grew more slowly than those formed in the presence of S alone, the numbers of colonies formed in the presence of each antibiotic were approximately equal within a 96-hour incubation period. In the presence of both antibiotics at these concentrations, very few colonies formed; they numbered approximately 1% or less of the number formed in the presence of the antibiotics used singly. A much higher proportion (15 to 25%) was obtained when the concentration of NV was lowered to 0.1 to 0.2 μg per ml. The concentration of S used was not so critical in the range 10 to 1000 μg per ml. Ten μg S per ml was selected for the *H. aegyptius* population since the bactericidal action of this concentration was less rapid than 100 or 1000 μg per ml in *H. influenzae* populations(8).

A high proportion of total transformants could form colonies in an environment con-

TABLE III. Linkage of Novobiocin (NV) and Streptomycin (S) Resistance Factors in *H. aegyptius*.

DNA		% transformants (μg S and [or] NV per ml)		
Transforming agent	% cell saturation*	S 10	NV .1	S 10 + NV .1
15 NV .4	100	0	.18	
	18	0	.032	
15 S 1000	100	.21	0	
	13	.028	0	
15 S 1000† + 15 NV .4	100	.12	.083	0
	33-39	.04	.032	.00007
15 S 1000‡ + 15 NV .4	100	.11	.09	.00034
	17-20	.022	.015	0
15 S NV .4	100	.16	.14	.025
	19-22	.035	.026	.0062

* "100% saturation" determined using DNA in excess of that required to produce maximal number of transformants. Approximately 0.5 μg DNA per ml used. Lower degrees of saturation calculated as follows:

$$\frac{\text{No. S or NV transformants at unsaturation level of DNA}}{\text{" " " " " " saturation " " "}} \times 100$$

Approximately 0.003 μg DNA per ml (final) used as unsaturated concentration.
† DNA concentration = that for DNAs used singly.
‡ DNA concentration = ½ that for DNAs used singly.

taining both antibiotics in the concentrations shown in Table III when the *H. aegyptius* 15 population was treated with TA 15 S NV 0.4. DNA concentrations, which were both above saturation and unsaturated with respect to cell concentration, were used. On the other hand, the low proportion of colonies formed in the presence of both antibiotics when the same population was treated with a mixture of the singly marked DNAs approximates that expected for unlinked genetic markers (frequency of double transformants is the product of the frequency of single transformants). The data of Table III thus indicate that NV resistance is linked to S resistance in *H. aegyptius* as well as in *H. influenzae*.

Examination of degree of linkage. The degree of genetic homology between *H. aegyptius* and *H. influenzae* was further explored by examining for the degree of linkage of the S and NV resistance factors in heterospecific and homospecific transformation. For this purpose, experiments were designed first to compare the *H. aegyptius* 15 and *H. influenzae* Rd populations for the rate and time required for phenotypic expression of the S and NV resistant transformants of both homospecific and heterospecific origin. An aliquot of a competent population of each receptor was exposed for 15 minutes to the homologous DNA directing both S and NV resistance. An aliquot was also exposed to the heterologous DNA carrying the S and NV resistance factors. After termination of the action of the DNAs by DNAase, the treated populations were appropriately diluted either into Levinthal broth and incubated at 37°C or diluted for pour plate preparations in Levinthal agar and incubated at 37°C. The pour plate preparations were incubated for 2 and 3 hours before layering with antibiotic containing agar (agar overlay method). Since the agar overlay method excludes measurement of progeny of fully-expressed transformants, it was used to define the incubation period beyond which there was no significant increase in numbers of transformants. DNA-treated populations diluted into Levinthal broth for incubation in the absence of antibiotics, were examined at 30-minute intervals for the number of S and NV transformants by removing aliquots and seeding them in pour plate preparations of agar containing the antibiotics. For the *H. influenzae* population, 1000 μg S per ml and 3 μg NV per ml were used to select the respective transformants and for the *H. aegyptius* population 100 μg S per ml and 0.2 μg NV per ml. The lower concentration of S (100 μg per ml) was adopted for the *H. aegyptius* 15 strain in order to have comparable degrees of dif-

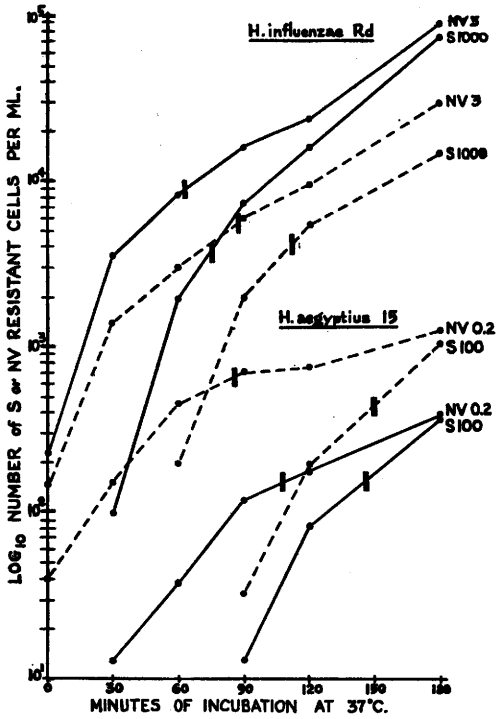

FIG. 1. Rate of phenotypic expression of heterospecific and homospecific DNA-mediated novobiocin (NV) and streptomycin (S) resistance in *H. influenzae* and *H. aegyptius*. *H. influenzae* Rd (upper curves) and *H. aegyptius* 15 (lower curves) incubated in broth for periods indicated before measurement of resistant cells. Solid bars on curves denote maximal number of transformants as measured by agar overlay method; increase in resistant cells beyond these points is interpreted as reproduction of transformed cells in broth. ●———●, TA Rd S NV 5; ●- - - -●, TA 15 S NV 0.4.

ference (300- to 500-fold) in concentration of the 2 antibiotics.

The results of one experiment for each receptor are represented in Fig. 1; a duplicate experiment yielded similar results. The data show that for the concentrations of the antibiotics selected, NV resistance is maximally expressed prior to S resistance in both receptor populations. This time difference in expression may be explained, however, by a bactericidal action of the high concentration of streptomycin. NV in the concentration used is bacteriostatic on the test populations within the time limit of the test. For example, populations of *H. influenzae* comparable in size to those used in the experiments of Fig. 1, are non-viable after a 10-minute exposure period to 100 μg S per ml(8); only approximately 50% of like sized populations of strain R*d* *H. influenzae* are non-viable after a 2-hour exposure to 3 μg of NV per ml. With *H. aegyptius* as test population, the DNA source appears to have less effect on the time at which the maximal number of transformants is expressed than with the *H. influenzae* strain as receptor.

The data of Fig. 1 show that for concentrations of the antibiotic selected, 2 hours' incubation prior to exposure to S and NV are adequate for expression of the maximal number of homospecific and heterospecific transformants when strain R*d* is the receptor. With strain 15 as receptor, a minimum of 2½ hours appears necessary for maximal expression of S resistant transformants. Since the generation time of strain 15 (approximately 45 minutes) is greater than that of strain R*d* (approximately 30 minutes), a difference in the time required for expression of the maximal number of transformants is expected.

The incubation period required after treatment with DNA for expression of the maximal number of transformants which are resistant to both antibiotics (linked transformants) was also measured by the above procedure using strain 15 as receptor. The same transforming agents were used but concentrations of antibiotics used to screen for linked transformants were lower: 10 μg S and 0.1 μg NV per ml. These concentrations were used to reduce the possibility of a synergistic type of action of the 2 antibiotics (see above). The incubation period required under these conditions for completion of transformation to resistance to S and NV in the same cell was similar to that needed for S resistant transformants alone ($>$2 hr$<$3 hr).

Competent *H. influenzae* R*d* and *H. aegyptius* 15 populations were then examined for degree of linkage in both heterospecific and homospecific transformation. The agar overlay method was used to measure the number of transformants. The data of Table IV indicate the degree of linkage for the transforming agents used at DNA concentrations below the level of cell concentration. The per cent of single transformants and those showing the linkage relationship were calculated on the basis of number of transformants in

TABLE IV. Degree of Linkage of Novobiocin (NV) and Streptomycin (S) Resistance Factors in *H. influenzae* and *H. aegyptius*.

Recipient population	DNA Source	DNA % cell saturation*	% transformants μg per ml S and [or] NV S 10	S 100	NV .1	NV 3	S + NV S 10 NV .1	S + NV S 100 NV 3	Ratio (as %) S·NV / S	Ratio (as %) S·NV / NV
H. influenzae Rd	Rd S NV 5	14		.42		.42		.13	31	31
	15 S NV .4	45		.29		.34		.10	35	29
H. aegyptius 15	Rd S NV 5	59	.09		.08		.02		22	25
	15 S NV .4	22	.05		.05		.01		20	20

Recipient populations tested simultaneously with each DNA.
* See Table III.

relation to total population. The class of single transformants includes those transformed to both S and NV resistant. Degree of linkage is interpreted as the ratio of number of transformants formed in the presence of the combined antibiotics, to number formed in the presence of each single antibiotic. Values of the ratios (Table IV) differ by approximately 30% for the 2 recipient populations but do not differ significantly with respect to the particular recipient population. Degree of linkage was also examined at the cell saturation level of DNA (approximately 0.5 to 1 μg DNA per ml). With strain R*d* *H. influenzae* as receptor, the ratios were comparable (range of 34 to 48%) for the 2 transforming agents. Populations of strain 15 *H. aegyptius* showed slightly lower values (range of 14 to 18%) after exposure to the *H. influenzae* DNA than after exposure to the homologous "species" DNA (range of 18 to 23%).

Discussion. The data presented demonstrate a natural "species" difference in sensitivity to novobiocin (NV) between *H. influenzae* and *H. aegyptius,* which is reflected by the *level* of NV resistance conferred in interspecific transformation; this reflection extends to transforming agents derived from spontaneous resistant mutant populations of differing degrees of resistance to NV. DNA from a strain of *H. influenzae* resistant to 0.5 μg NV per ml (but not 2 μg per ml), conferred a 5- to 10-fold lower level in the *H. aegyptius* population than in the *H. influenzae* population. Similarly, transforming agents derived from spontaneous mutant populations resistant to much higher levels of NV (*H. influenzae,* 30 μg per ml; *H. aegyptius,* 6 μg per ml), conferred significantly different levels (approximately 10-fold) in sensitive populations of the 2 "species" (data to be published). Also, *intra*specific transformations may reflect natural differences in sensitivity to NV of different types of *H. influenzae* as shown in Table II for strains R*b* and R*d* *H. influenzae*. These observations suggest that a genetic factor(s) (modifier gene(s)) inherent in the receptor population influences the level of NV resistance to be associated with DNA-conferred NV resistance. This factor(s) does not appear to influence streptomycin (S) resistance; no significant difference in natural sensitivity or DNA-conferred resistance to streptomycin was noted for the 2 "species." The genetic traits conditioning resistance to antibiotics other than NV and S have not been examined for this factor, but Davis *et al*(9) report that *H. aegyptius* strains are more sensitive to antibiotics in general than *H. influenzae* strains. It was not possible to demonstrate resistance to concentrations of NV as low as 0.05 μg per ml after exposure of *H. aegyptius* populations to the DNA from *H. influenzae* cells normally sensitive to NV. Therefore, it may not be possible to confer by transformation the factor(s) which controls the greater normal resistance in *H. influenzae* populations. On the other hand, the difference in natural sensitivity of these 2 "species" may be the absence (or presence in reduced amounts) of a factor(s) in *H. influenzae* populations which is present to a significant degree in *H. aegyp-*

tius populations. Modifier genes which influence the level of streptomycin resistance conferred in pneumococcal and streptococcal interspecific transformation have been reported by Ravin and De Sa(10).

It may be inferred from the data presented that spontaneous mutation of strain R*d* *H. influenzae* to resistance to 5 μg NV per ml and strain 15 *H. aegyptius* to 0.4 μg NV per ml represents a similar mutational step at the NV locus, since transforming agents (DNA) derived from mutant populations with these degrees of resistance confer a similar level of resistance in a particular recipient population (Table II). The evidence that the degree of linkage of the 2 markers (S and NV resistance) is similar in homospecific and heterospecific transformation suggests that, at least for the transforming agents used, similarity in structure or specificity of the DNA extends not only to the mutational step at the NV locus, but also to the DNA molecule (or part) which encompasses both genetic markers.

A difference in the action of the 2 transforming agents used in the experiments reported has, however, been noted. Reference to Table II shows that a higher proportion of transformants is obtained at some of the higher concentrations of NV employed when the transforming agent from the *H. influenzae* strain (TA R*d* S NV 5) is used. Subsequent examination of the action of this particular transforming agent in sensitive *H. aegyptius* populations has shown that colonies which form in the presence of 1 μg NV per ml are virtually all streptomycin resistant. This selection at the higher NV concentrations for "linked transformants" in the *H. aegyptius* population is under study; it appears to explain, in part at least, data which suggested that a higher level of NV resistance (approximately 1 μg per ml) could be conferred in the *H. aegyptius* population by a step-wise exposure to the *H. influenzae* transforming agent (TA R*d* S NV 5).

Summary. DNA-mediated transformation to novobiocin resistance has been conferred in sensitive *H. aegyptius* populations. A comparison of the frequency of this event induced by DNA of heterologous source (resistant R*d* *H. influenzae*) and by DNA of homologous source (resistant *H. aegyptius*) supports the earlier report(3) which suggested a high degree of genetic homology between *H. aegyptius* and *H. influenzae.* The data suggest that a genetic factor(s) inherent in the receptor population influences the level of novobiocin resistance conferred by a given DNA. The genetic factors controlling streptomycin and novobiocin resistance have been demonstrated to be linked in *H. aegyptius.*

1. Ravin, A. W., Advances in Genetics, 1961, v10, 61.

2. Leidy, G., Hahn, E., Alexander, H. E., J. Exp. Med., 1956, v104, 305.

3. ———, Proc. Soc. Exp. Biol. and Med., 1959, v102, 86.

4. Goodgal, S. H., J. Gen. Physiol., 1961, v45, 205.

5. Goodgal, S. H., Herriott, R. M., ibid., 1961, v44, 1201.

6. Alexander, H. E., Bacterial and Mycotic Infections of Man, R. J. Dubos, ed., J. B. Lippincott Co., 1958, 3rd ed.

7. Leidy, G., Jaffee, I., Alexander, H. E., Proc. Soc. Exp. Biol. and Med., 1962, v111, 725.

8. Alexander, H. E., Leidy, G., Pediatrics, 1949, v3, 277.

9. Davis, D. J., Pittman, M., Vogel, J. E., Ottinger, B., Antibiot. & Chemother., 1955, v5, 363.

10. Ravin, A. W., De Sa, J. D. H., J. Bacteriol., 1964, v87, 86.

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